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**TITLE: PRODUCTION OF ENZYMATICALLY ACTIVE HUMAN
ACETYLCHOLINESTERASE IN E. COLI**

**SUBTITLE: Expression and Reconstitution of Biologically
Active Human Acetylcholinesterase from E. Coli**

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SUMMARY

Authentic human acetylcholinesterase (hAChE) was expressed in *Escherichia coli* under regulation of the constitutive *deo* promoter or the thermoinducible λP_L promoter. To facilitate the expression in the prokaryotic system, the recombinant human AChE (rhAChE) cDNA was modified at the N-terminus by site-directed mutagenesis in order to replace some of the GC-rich regions by AT. These modifications did not alter the amino acid sequence but resulted in ample production of the protein. rhAChE accumulated in the cells and reached a level of 10% of total bacterial proteins. A partially purified inactive recombinant protein was recovered from inclusion bodies. Active rhAChE was obtained after solubilization, folding and oxidation, although the recovery of the active enzyme was low. A 20–40-fold increase in rhAChE enzyme activity was achieved by replacing Cys⁵⁸⁰ by Ser. Substrate specificity and inhibitor selectivity of the recombinant mutant enzyme were indistinguishable from native AChE isolated from human erythrocytes.

Preliminary results of this research were presented at the *Proceedings of the 1991 Medical Defense Bioscience Review*, 7–8 August 1991, Edgewood, Maryland. A paper summarizing these findings entitled "Expression and Reconstitution of Biologically active Human Acetylcholinesterase from *E. Coli*" by M. Fischer, A. Ittah, I. Liefer and M. Gorecki was accepted for publication in *Cellular and Molecular Neurobiology*.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is present in the neuromuscular junctions and brain cholinergic synapses and plays a pivotal role in neurotransmission by degrading acetylcholine, resulting in termination of muscle or nerve stimulation. The AChE is sensitive to inhibition by a number of tertiary amines and organophosphates, which *in vivo* can cause severe impairment and death. For example, AChE is the primary target of military "nerve gases" and agricultural insecticides. The therapeutic potential of exogenous AChE has provoked considerable interest. Acetylcholinesterase and butyrylcholinesterase (BuChE) have been shown, in several animal studies, to protect mice against lethal dosages of soman or 7-(methyl-ethoxyphosphonyloxy-1-methylquinolinium iodide (MEPQ) by intraperitoneal administration of the enzyme prior to exposure to the toxic agent (Raveh et al. 1989, Ashani et al. 1991). However, more extensive evaluation of the clinical benefit of AChE has been hindered by limited availability.

Recently, a cDNA prepared from adult basal ganglia cells encoding hAChE catalytic subunit was isolated and cloned (Soreq et al. 1990). The deduced amino acid sequence of the mature enzyme is 583 residues in length and contains three putative glycosylation sites. Synthetic mRNA, generated from the cDNA *in vitro*, was translated in microinjected oocytes into catalytically active enzyme. The enzyme produced in oocytes exhibits biochemical properties similar to the mature enzyme as manifested by substrate inhibition and sensitivity to the specific AChE inhibitor BW284C51 (Augustinsson 1963).

The same cDNA was used to transiently express hAChE in embryonic kidney cell line 293. The recombinant enzyme was enzymatically active and exhibited biochemical properties similar to the native enzyme (Valen et al. 1991a). The recombinant enzyme secreted into the medium contained three molecular forms, monomers, dimers and tetramers in equimolar ratio.

In this midterm report, we describe the construction of expression plasmids that support high-level production of rhAChE in *E. Coli* and the in vitro reconstitution of the inactive protein from inclusion bodies into enzymatically active AChE. The in vitro reconstituted enzyme is a monomer and possesses biochemical properties which are indistinguishable from native AChE in terms of substrate specificity and inhibitor selectivity.

EXPERIMENTAL PROCEDURES

Reagents

Acetylthiocholine, HEPES, 5,5-Dithiobisnitrobenzoic acid (DTNB), tetraisopropylpyrophosphoreamide (iso-OMPA), 1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284C51), Tris, urea, glutathione-oxidized (GSSG), polyethyleneglycol (PEG), L-arginine, tetramethylammoniumchloride (TMAC), ethylenediaminetetracetic acid (EDTA), acrylamide, sodium dodecylsulfate (SDS), Coomassie Blue, butyrylthiocholine, ampicillin (Amp), tetracycline (Tet) were purchased from Sigma Chemical Co. St-Louis MI, U.S.A. Guanidinethiocyanate (GTC) was from Fluka Chemical AG, Switzerland, DEAE-Sepharose and cyanogenbromide activated Sepharose 4B from Pharmacia (Sweden). 1-methyl-9[NB-(ϵ -aminocaproyl)- β -aminopropylamino] acridinium bromide (MAC) was obtained from the Weizmann Institute, Rehovot, Israel.

Enzymes: Restriction endonucleases, T_4 -ligase, polynucleotide kinase, E. Coli DNA polymerase (Klenow fragment) were purchased from New England Bio-Labs Inc. (MA, U.S.A.). Erythrocyte derived hAChE and lysozyme were from Sigma Co, (U.S.A.)

Equipment: Enzyme activities, protein and turbidimetric determinations were done using PU 8700 UV/visible spectrophotometer (Phillips). Fraction collector and monitoring units were from Pharmacia, Sweden. Densitometric analysis was made with Model 620 Vidio densitometer Bio-Rad Laboratories Inc.

Construction of expression vector: A 4Kb DNA fragment harboring the hAChE was isolated from plasmid GEM-7 (Soreq et al. 1990, harboring the DNA sequences shown in Figure 8) kindly provided by Prof. H. Soreq (The Hebrew University of Jerusalem). The 2200bp AChE sequence that resides on the distal part of the 4Kb segment and is flanked by EcoRI-XhoI sites was isolated and ligated into pBR322 cleaved with EcoRI-SalI restriction endonucleases. The resulting plasmid containing the AChE was cleaved with NdeI and NaeI and the larger of the two fragments generated by the cleavage was isolated from the agarose gel. A synthetic oligonucleotide containing complementary restriction sites (Figure 1) was ligated to the purified fragment. The synthetic oligonucleotide contained the initiation codon ATG and the sequence of AChE starting from base number 253 to base 298 (Soreq et al. 1990) which corresponds to the beginning of the mature protein after processing of the sequences encoding leader peptide upstream of base number 253. The resulting plasmid pAIF-2 (Figure 1A) was cleaved with AatII (at base number 2128), made blunt end by DNA polymerase (Klenow fragment) and then cleaved with NdeI. The 1875bp fragment generated by these restriction enzymes was purified from agarose gel and ligated into appropriate expression vectors (not shown). In these constructs we have placed the hAChE sequence under control of the constitutive deo P promoters (Fischer et al. 1990) or the thermoinducible λP_L promoter (Hartman et al. 1986). The corresponding expression plasmids containing the authentic GC-rich stretches in the 5'-end of the hAChE-DNA sequence were designated pAIF-4 and pAIF-11, respectively (Figure 1A).

Manipulation of the 5'-end GC-rich sequence: The GC-rich sequence at the 5'-end of the hAChE was substituted with A or T in degenerated codons such that the amino acid sequence was not altered (Figure 1B). A synthetic oligonucleotide flanked by NdeI-NcoI containing 24 base substitution was ligated to pAIF-4 and pAIF-11. The resulting expression plasmids, driven by the thermoinducible and constitutive promoters, were designated pAIF-34 and pAIF-51, respectively. In plasmid pAIF-51 the Amp^R gene was replaced with the Tet^R gene to yield pAIF-52 (Figure 2). Replacement of Amp^R by Tet^R gene in the expression vector pAIF-52 stabilized the plasmid and expression of rhAChE was maintained at the same level for more than 40 generations.

Substitution of Cys⁵⁸⁰ by Ser: Three intrasubunit disulfide bonds can be deduced from the DNA sequence (Soreq et al. 1990) and from the three-dimensional structure of the Torpedo AChE (Sussman et al. 1991). The C-terminus cysteine residue of Torpedo AChE participates in the intersubunit disulfide bridge between two subunits (MacPhee-Quigley et al. 1986). Since the C-terminus cysteine residue of human and Torpedo AChE are positioned three amino acids upstream of the last amino acid we predicted that Cys⁵⁸⁰ (the C-terminus) of hAChE is involved in generating a disulfide bridge between two subunits and not required for maintaining the protein integrity. Thus, this cysteine was replaced with serine by cleavage of plasmid pAIF-52 with SacI and XbaI and insertion of a synthetic oligonucleotide flanked by complementary sites that contained the serine codon TCA instead of the cysteine codon TGC in the authentic sequence. The resulting plasmid was designated pMFL-52Ser (Figure 2). Bacterial hosts and plasmid constructs are presented in Table 1.

Media and growth: Cultures were grown in LB supplemented with M9 salts and 0.1% glucose, 100µ/ml ampicillin or 12µg/ml tetracycline depending on the culture used. Clones SØ930pMFL-52Ser and SØ930pAIF-52 were grown at 30°C for 16–18h, harvested by centrifugation for 10 min. at 10,000 RPM in Sorval refrigerated centrifuge, washed with 50mM Tris-HCl, pH 8.0. and inclusion bodies were prepared as described below. A4255 pAIF-34 (the thermoinducible expression system) was grown to OD₆₆₀ of 0.8 at 30°C. To induce expression of rhAChE, the temperature was elevated to 42° and grown for an additional 2h. The culture was harvested by centrifugation in Sorval refrigerated centrifuge for 10 min at 10,000 RPM. Cell lysates containing about 600µg of protein in 100µl were prepared by NaOH-SDS lysis solution (Hartman et al. 1986), boiled for 10 min and 15µl was applied per slot of SDS-PAGE. Following electrophoresis at room temperature the gel was stained with Coomassie Blue R.

Table 1:

Plasmid constructs

Plasmid	Resistance Marker	Promoter upstream to AChE	Ribosomal binding site	AChE sequence modifications
pAIF-2	Amp	none	none	none
pAIF-4	Amp	λP_L (thermoinducible)	λcII	none
pAIF-11	Amp	deoP (constitutive)	deo	none
pAIF-34	Amp	λP_L	λcII	24 G and C to A and T (Figure 1)
pAIF-51	Amp	deoP	deo	24 G and C to A and T
pAIF-52	Tet	deoP	deo	24 G and C to A and T
pMFL-52Ser	Tet	deoP	deo	As pAIF-52 and cys ⁵⁸⁰ -Ser
pMIF-35Ser*	Amp	λP_L	λcII	As pAIF-34 and cys ⁵⁸⁰ -Ser
E. Coli Hosts				
Strains	Genotype			
A4255	λcI_{857} , ΔH_1 BamH, bio			
SØ930	Δdeo , $deoR-7$, $clmA$, Δlac , udp , upp , ton , thi . (Fischer et al. 1990)			

* The marked construct was not analyzed.

Inclusion body purification and solubilization: To isolate inclusion bodies, 10g of packed cells were suspended in 100ml of 50mM Tris-HCl, pH 8.0, 10mM EDTA and 10 μ g/ml of lysozyme. The suspension was incubated for 2h at room temperature, sonicated intermittently for 5 min to disrupt cells and centrifuged for 15 min at 15,000 rpm in Sorval refrigerated centrifuge. The pellet was resuspended in 100ml of distilled water and stirred for 30 min. After centrifugation, the pellet was suspended in 10mM Tris-HCl, pH 8.0, containing 4M urea and stirred at room temperature for 1h. The inclusion bodies were collected by centrifugation for 30 min at 15,000 rpm, resuspended in 100ml distilled water and stirred at 4°C for 16–18h. Inclusion bodies were then collected by centrifugation and resuspended in 10ml of 10mM Tris-HCl, pH 8.0. Solid guanidinetiocyante (GTC) was added to a final concentration of 5.5M in 25ml. After solubilization of inclusion bodies, the solution was brought to pH 8.6 with 1M NaOH and was allowed to stir for 16–18h at room temperature. The GTC solubilized inclusion bodies were centrifuged at 15,000 rpm for 30 min at room temperature to remove undissolved matter and then were diluted 1:10 into 10mM Tris-HCl, pH 8.6, containing 8M urea.

Reconstitution of rhAChE in vitro: The 1:10 diluted rhAChE in 8M urea was diluted into refolding solution to yield a final concentration of 30–100 μ g/ml protein. The refolding solution contained 0.5M L-arginine, pH 10.0, 1M tetramethylammonium chloride, 0.3mM GSSG and 0.3% polyethyleneglycol 4000 at 4°C. After the addition of denatured rhAChE, the solution was incubated for 24–72h at 4–8°C, dialyzed against 5mM L-arginine, pH 10.0, containing 1mM EDTA, for 16–18h at room temperature. Acetylcholinesterase activity was determined according to Ellman et al. (1961) at 412nm using acetylthiocholine as substrate. The assay mixture contained 0.1M HEPES, pH 8.0, instead of phosphate buffer. We have introduced this modification because no spontaneous hydrolysis of acetylthiocholine is observed in HEPES.

Purification of active rhAChE: The active rhAChE was subjected to Q-Sepharose column chromatography. The column was equilibrated with 20mM HEPES, pH 8.0, and the active rhAChE

was eluted with NaCl gradient. Fractions containing active AChE were pooled and precipitated with ammonium sulfate at 45% saturation, dialyzed against 20mM HEPES, pH 8.0, and applied to MAC-Sepharose 4B prepared according to Dudai et al. (1972). Active rhAChE was eluted from the affinity matrix with 20mM HEPES, pH 8.0, containing 2M NaCl.

Standard procedures: DNA sequencing was performed according to the dideoxynucleotide incorporation by the method of Sanger et al. (1977). Restriction endonuclease cleavage and ligation were done according to the manufacturer's recommendations. SDS-PAGE was according to Laemmli (1970). Protein determination was according to the procedure described by Bradford (1976). AChE activity gels were performed as described by Karnovsky and Roots (1964). Amino acid sequence was determined with Protein Microsequencer (Applied Biosystem 475A) based on the Edman degradation procedure (Edman 1950; Tarr 1977).

RESULTS

Expression of rhAChE in E. Coli: Preliminary attempts to express hAChE gene by two different expression vectors, pAIF-4 and pAIF-11, under control of the λP_L or the deoP promoters respectively, were unsuccessful. The GC-rich sequences at the 5'-end of the gene might have generated secondary structures in the mRNA that are poorly accessible to ribosomes. Therefore, the high GC content at the 5'-end of the sequence, was replaced by A or T at "wobble" positions which do not change the authentic amino acid sequence, as shown in Figure 1. Expression vectors harboring the deoP and the λP_L promoters and the modified sequence were introduced into E. Coli SØ930 and A4255, respectively. Total cell lysates prepared in SDS-NaOH were analyzed on SDS-PAGE. Figure 3 shows that an intense protein band corresponding to a molecular weight of 62kD accumulates in the induced culture of A4255 pAIF-34 (lane 2). This protein band is not seen in uninduced control culture (Figure 3, lane 1). Western blot analysis, presented in Figure 3A, revealed that the 62kD protein band immunoreacted with antibodies prepared against erythrocyte-derived authentic AChE and thus confirmed that the newly produced 62kD protein band represents a genuine AChE polypeptide. Densitometric analysis of lane 2 (Figure 3) aimed to determine expression level show that the 62kD protein band, peak number 9, represent about 10% of the total cell protein. A similar expression level of rhAChE protein was obtained in clone SØ930pMFL-52Ser (Figure 3B). In view of the simpler growth conditions for expression of rhAChE clones harboring the deo promoters driven system we elected to use it routinely.

To determine the localization of expressed AChE, the A4255 pAIF-34 and SØ930pMFL-52Ser cultures were sonicated until 99% of the cells were disrupted and the soluble fraction was separated from insoluble matter by centrifugation. The nonsoluble fraction was resuspended in 1% SDS-NaOH and 5 μ g of protein of each fraction was analyzed on SDS-PAGE. The electrophoretic pattern revealed that rhAChE was localized in inclusion bodies in both cultures (Figure 3, lane 4 and Figure 3B lane 3) and did not possess enzymatic activity. The 62kD protein band, observed on SDS-PAGE,

was isolated and the amino acid sequence of the N-terminus was determined. The results of the sequencing confirmed the expected sequence of Met-Glu-Gly-Arg-Glu-Asp-Ala-Glu-Leu-Leu-Val.

Enzyme activity of wild-type and mutant (Cys⁵⁸⁰-Ser⁵⁸⁰) rhAChE: Solubilized inclusion bodies purified from SØ930pAIF-52 and SØ930pMFL-52Ser cultures were refolded and oxidized to obtain enzymatically active protein as described in Experimental Procedures. Table 2 compares the rhAChE activities obtained with construct containing the native amino acid sequence and with mutant in which Cys⁵⁸⁰ was replaced by Ser. The enzyme activity obtained with the Cys⁵⁸⁰-Ser⁵⁸⁰ mutant was considerably higher than that obtained with the wild-type. A 71-fold increase in specific activity of the mutant AChE was obtained relative to the wild-type in the absence of GSSG, whereas a 32-fold higher specific activity of the mutant protein was noted in the presence of GSSG. The increase in recovery of enzyme activity after in vitro refolding of the mutant rhAChE was apparently related to the lower number of Cys residues in the molecule.

Table 2:

Activity of rhAChE after refolding and oxidation

rhAChE	GSSG	Activity (u/ml)	Protein (mg/ml)	Specific Activity (u/mg protein)
Native	-	0.007	0.025	0.28
Native	+	0.040	0.030	1.40
Mutant	-	0.580	0.029	20.10
Mutant	+	1.200	0.026	46.10

Effect of mutation on activity of rhAChE after refolding and oxidation. Inclusion bodies purified from SØ930pAIF-52 that produce rhAChE with Cys⁵⁸⁰ (native) and SØ930pMFL-52Ser that produce rhAChE with Ser⁵⁸⁰ (mutant), were solubilized in 5.5M guanidinetiocyante and subjected to renaturation in L-arginine refolding solution for 48h at 4-6°C with and without GSSG. Enzyme activity was determined after removal of salts by dialysis. One unit equals to amount of enzyme which hydrolyzes 1 µmole of acetylthiocholine per min. at 25°C. Accuracy of protein measurement was ± 5% and of activity ± 2%.

The zymogram of rhAChE on nondenaturing 6% polyacrylamide gel stained for enzymatic activity according to Karnovsky and Roots (1964) is shown in Figure 3C. The zymogram revealed that the activity band generated by the native enzyme migrated slightly faster relative to the mutant harboring the Cys⁵⁸⁰-Ser⁵⁸⁰ substitution.

Partial purification of rhAChE: Since higher AChE activity was obtained with the mutant enzyme, we have focused on the purification of this analogue. One liter of reconstituted rhAChE containing 1260 units was concentrated and dialyzed using a "Pellicon" dialysis concentrator and a 30kD MW cutoff membrane. The volume was reduced to 600ml in 20mM HEPES, pH 8.0, and applied to a Q-Sepharose column chromatography.

Table 3 summarizes the results of purification. Pooled fractions, containing AChE activity, were eluted from Q-Sepharose at about 0.275-0.375mM NaCl. Calculations of yield showed that 64% of the activity was recovered with a specific activity of 117u/mg. After precipitation with 45% saturation of ammonium sulfate and dialysis against 20mM HEPES, pH 8.0, for 180 units were applied to MAC-Sepharose 4-B affinity column (1ml bed volume). As shown in Table 3, the affinity chromatography step improved purity by 19-fold, with an overall recovery of 84%. SDS-PAGE analysis of purified rhAChE revealed a single protein band on Coomassie stained gel (Figure 4), and indicates that the rhAChE was purified to homogeneity.

Table 3:**Purification of rhAChE**

	Volume (ml)	Activity (units)	Protein (mg)	Specific Activity (u/mg protein)
Q-Sepharose load	600	1260	30	42
Q-Sepharose eluate	23	805	7.8	117
MAC-Sepharose load	1	180	1.5	120
MAC-Sepharose eluate	10	151	0.066	2289

Inclusion bodies derived from SØ930pMFL-52Ser were solubilized and subjected to in vitro refolding. Following concentration-dialysis, rhAChE was first subjected to Q-Sepharose ion-exchange chromatography. The pool of active fractions, eluted by NaCl gradient, was processed and subjected to MAC-Sepharose 4B affinity chromatography. Note that only 180 out of 805 units were loaded onto MAC-Sepharose affinity column due to the limiting capacity of the small bed volume. After repetition of the affinity step several times to process the entire batch of 805 units, active fractions were pooled, concentrated by ammonium sulphate precipitation (45% saturation), resuspended in 1ml of 20mM HEPES, pH 8.0, 2.5mM EDTA and dialyzed against 4 liters of the same buffer. A total of 0.290mg protein containing 661 units of active rhAChE was recovered (52.5% yield by activity).

Biochemical characterization of rhAChE: Km values for acetylthiocholine and butyrylthiocholine for the Ser⁵⁸⁰ mutant rhAChE were calculated by the method of Lineweaver and Burk (Figure 5). The apparent Km obtained with acetylthiocholine was 0.1158±0.0067mM (three independent determinations), while with butyrylthiocholine, the Km was 12.5±0.7mM that is more than 100-fold higher. The Km of the crude recombinant AChE, derived from clone SØ930pAIF-52 harboring the native amino acid Cys⁵⁸⁰, was 0.125mM for acetylthiocholine (Figure 5A) and indicates that the

substitution of Cys⁵⁸⁰ to Ser did not alter the catalytic properties.

To define further the properties of the mutant rhAChE, its inhibition by the AChE specific inhibitor BW284C51 and the BuChE specific inhibitor iso-OMPA were compared (Figure 6). The sigmoid profiles of inhibition obtained for the *in vitro* refolded enzyme and the native erythrocyte AChE are quite similar. 50% inhibition was obtained at 1nM of BW284C51 for both enzymes. A 50% inhibition with iso-OMPA was obtained at 0.9mM which is six orders of magnitude higher relative to the inhibitory concentration of BW284C51, and is comparable with results obtained for native AChE.

The subunit character of the mutant rhAChE was determined by gel permeation chromatography on Sephacryl 300. The column was equilibrated with 10mM L-arginine, pH 10.0, and 50mM NaCl. Bovine serum albumin containing the 67kD monomer and the 135kD dimer was used as an internal control. About 50 units of the highly purified recombinant enzyme was mixed with BSA and applied to the column. Figure 7 shows that the 135kD BSA dimer eluted at fraction 45 and the 67kD monomer eluted at fraction 50, while the activity of the active rhAChE peaked in fraction 53. Similar analysis by HPLC with Superose 12 column consistently resulted in elution of the 67kD BSA monomer prior to the elution of active rhAChE (data not shown). These results indicate that the *in vitro* reconstituted active rhAChE is a monomer.

DISCUSSION

The DNA sequence encoding the human AChE catalytic subunit was inserted into *E. Coli* expression vectors under control of λP_L or the *E. Coli* *deo* promoters. Cultures harboring these vectors failed to produce the cloned gene product as determined by SDS-PAGE or Western blot analysis. The lack of rhAChE expression in clones A4255pAIF-4 and SØ930pAIF-11 is attributed to the high GC content of the cloned gene flanking the ribosomal binding site. GC-rich sequences in mRNA in the vicinity of the ribosomal binding site often generate stem-loop structures which may block translation (Kozak 1983, Looman et al. 1986). Disruption of the GC-rich sequences at the 5'-end of the cloned hAChE indeed resulted in high expression levels of rhAChE in clones A4255pAIF-34 and SØ930pAIF-52, driven by λP_L and *deo* promoters, respectively.

The rhAChE expressed in *E. Coli* accumulates as an aggregate in inclusion bodies, as observed for a number of other cloned eukaryotic genes in *E. Coli* (Mitraki and King 1989), and possesses no AChE activity. Solubilization and refolding of the 62kD polypeptide into enzymatically active form was achieved in the presence of L-arginine and GSSG, which were shown effective in refolding of Fab fragment produced in *E. Coli* (Buchner and Rudolph 1991). The attainable enzyme activities reconstituted *in vitro* and derived from clones SØ930pAIF-52 were low (Table 3). We suspected that the odd number of seven cysteine residues in the hAChE (Soreq et al. 1990) enhanced incorrect disulfide bond formation during refolding. Indeed, replacement of cysteine 580 by serine resulted in considerably higher yield of active rhAChE upon refolding (Table 3). The specific activity of reconstituted enzyme prepared from inclusion bodies produced in SØ930pMFL-52Ser ranged between 35 and 56 units/mg protein.

The data obtained from the gel permeation chromatography of highly purified rhAChE (2289 u/mg protein) reveal that the active enzyme is a monomer. Valen et al. (1991b) have recently shown that replacement of Cys⁵⁸⁰ with Ala resulted in secretion of predominantly monomeric hAChE from

transiently transfected human embryonic kidney cell line 293. The active rhAChE monomer produced in tissue culture differs from rhAChE from *E. Coli* in that the latter enzyme is not glycosylated since *E. Coli* does not harbor a glycosylating system. Hence glycosylation appears to be not essential for catalytic activity.

The catalytic properties of the reconstituted rhAChE, as demonstrated by substrate specificity and selective inhibition by BW284C51 and iso-OMPA, are quite similar to those of native AChE derived from erythrocytes (Figure 6) and the rhAChE expressed in tissue culture (Valen et al. 1991a, 1991b). The *E. Coli*-derived enzyme and the erythrocyte AChE show a 50% inhibition at 1nM BW284C51 while the enzyme produced in the eukaryotic expression system was inhibited at 8nM (Valen et al. 1991a). The 50% inhibitory concentration of iso-OMPA is in the 100–200mM range for the native and the recombinant AChE and further elucidates the "true" nature of the *E. Coli*-derived enzyme.

The bacterial expression system and the *in vitro* reconstitution of rhAChE opens the possibility to prepare large quantities of the enzyme to be tested as a potential therapeutic and prophylactic agent against organophosphates.

Task and achievements

Contractor tasks have been defined in USAMRDC log No. 68351002 dated June 22, 1990. Table 4 summarizes the achievements by tasks.

Table 4

Tasks	Status
1. Preparation of full-length cDNA clone	Full length cDNA was provided by Prof. H. Soreq
2. Construction of AChE expression vectors 2.1 Termoducible (λ P _L driven) 2.2 Constitutive (deo promoter driven)	Completed
3. Optimization of AChE expression and scale-up fermentation 3.1 λ P _L expression system 3.2 Constitutive expression system	In progress
4. Development of an AChE purification scheme 4.1 Assessment of biological activity 4.2 Reconstitution of inactive AChE to enzymatically active form 4.3 Purification process to obtain highly purified rhAChE	In progress Completed Completed To be completed
5. Characterization of recombinant AChE 5.1 M.W. determination of rhAChE 5.2 Effect of reducing agent 5.3 Western analysis 5.4 Enzymatic activity in solution and nondenaturing gels 5.5 Substrate and inhibitor specificity ----- 5.6 Final recombinant AChE product characterization 5.6.1 Isoelectric focusing 5.6.2 Two dimensional 5.6.3 Peptide mapping 5.6.4 Amino acid analysis 5.6.5 Amino acid sequencing 5.6.6 U.V. absorption spectroscopy 5.6.7 Fluorescence emission spectroscopy 5.6.8 Circular dichroism 5.6.9 Extinction coefficient 5.6.10 Stability	Completed To be determined Completed Completed Completed ----- All to be determined in the future
6. Scale-up purification process and production of rhAChE	To be developed in the future

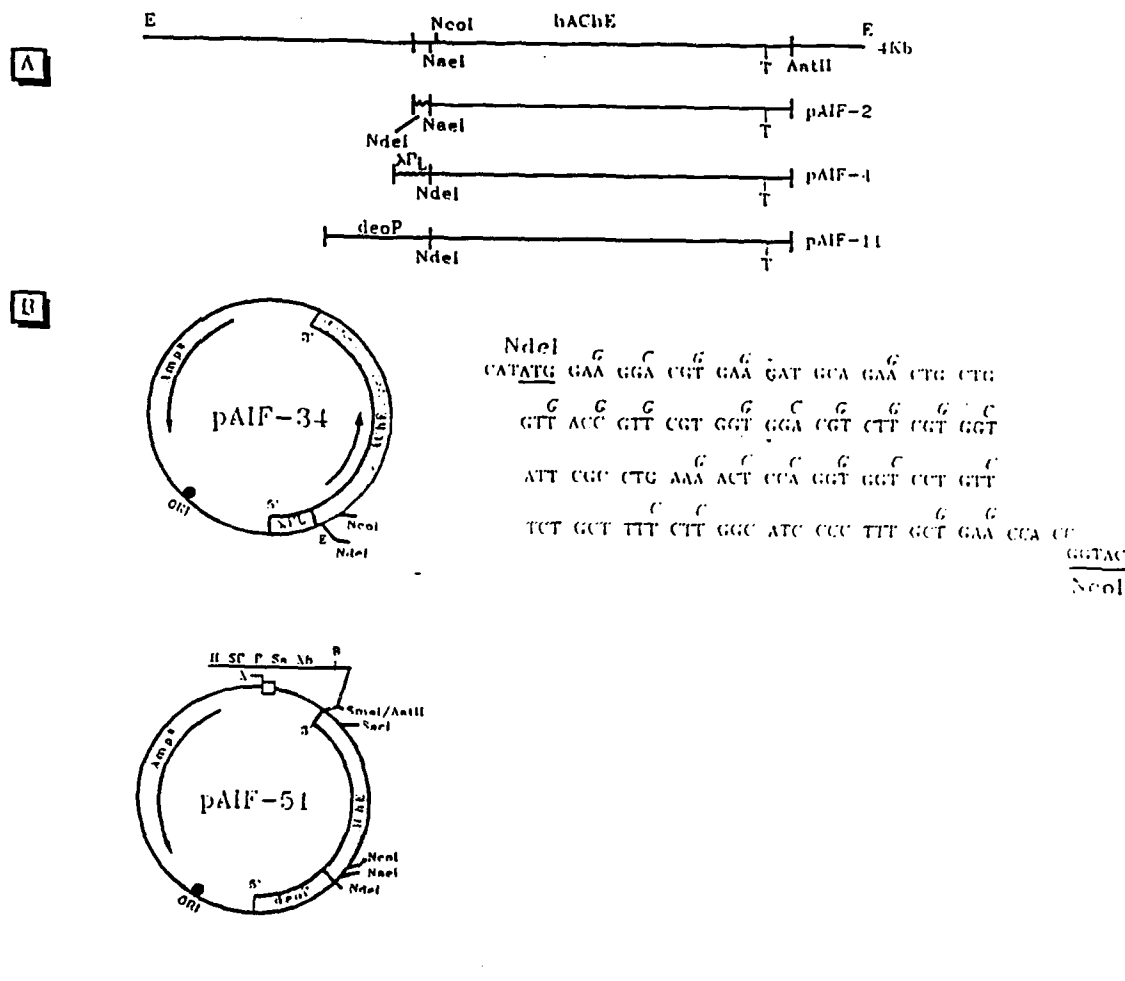


Figure 1: Schematic presentation of rhAChE harboring plasmids.

- A:** From top to bottom: A linear presentation of the coding sequences for AChE and respective promoters cloned in pBR322. 4Kb; fragment harboring the hAChE cDNA. pAIF-2; intermediate construct which contains a synthetic linker harboring ATG translation initiation codon and the authentic, GC-rich, DNA sequence starting with the triplet GAG, encoding Glu, the first amino acid of mature hAChE protein. pAIF-4 and pAIF-11 are expression vectors driven by the thermoinducible λP_L and the constitutive deoP promoter, respectively. The NdeI site (CATATG) contain translation initiation codon (underline). T, indicates translation stop codon located 120bp upstream to AatII.
- B:** pAIF-34 and pAIF-51 plasmid with either λP_L or deoP promoters and a synthetic oligonucleotide replacing sequence between NdeI-NcoI site. The synthetic oligonucleotide sequence is shown on the right. The G C (with top letters) nucleotides substituted by A T. Restriction cleavage site E, H, Sp, P, Sa, Xb, B, and X are EcoRI, HindIII, SspI, PstI, Sall, XbaI, BamHI and XhoI, respectively.

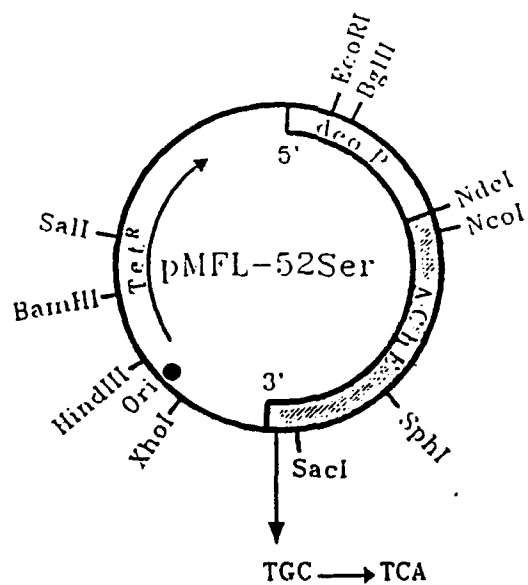
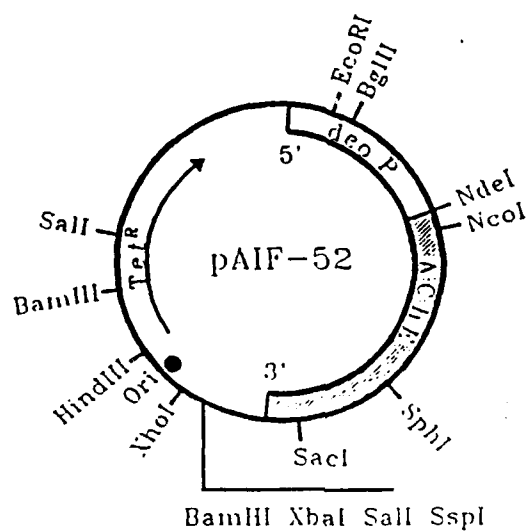


Figure 2: Schematic presentation of expression plasmids. pAIF-52 contains the native amino acid sequence. pMFL-52Ser is identical to pAIF-52 with the exception that Cys⁵⁸⁰ was replaced by Ser. The three-letter codon for cysteine, TGC, is replaced by the three-letter codon for serine, TCA.

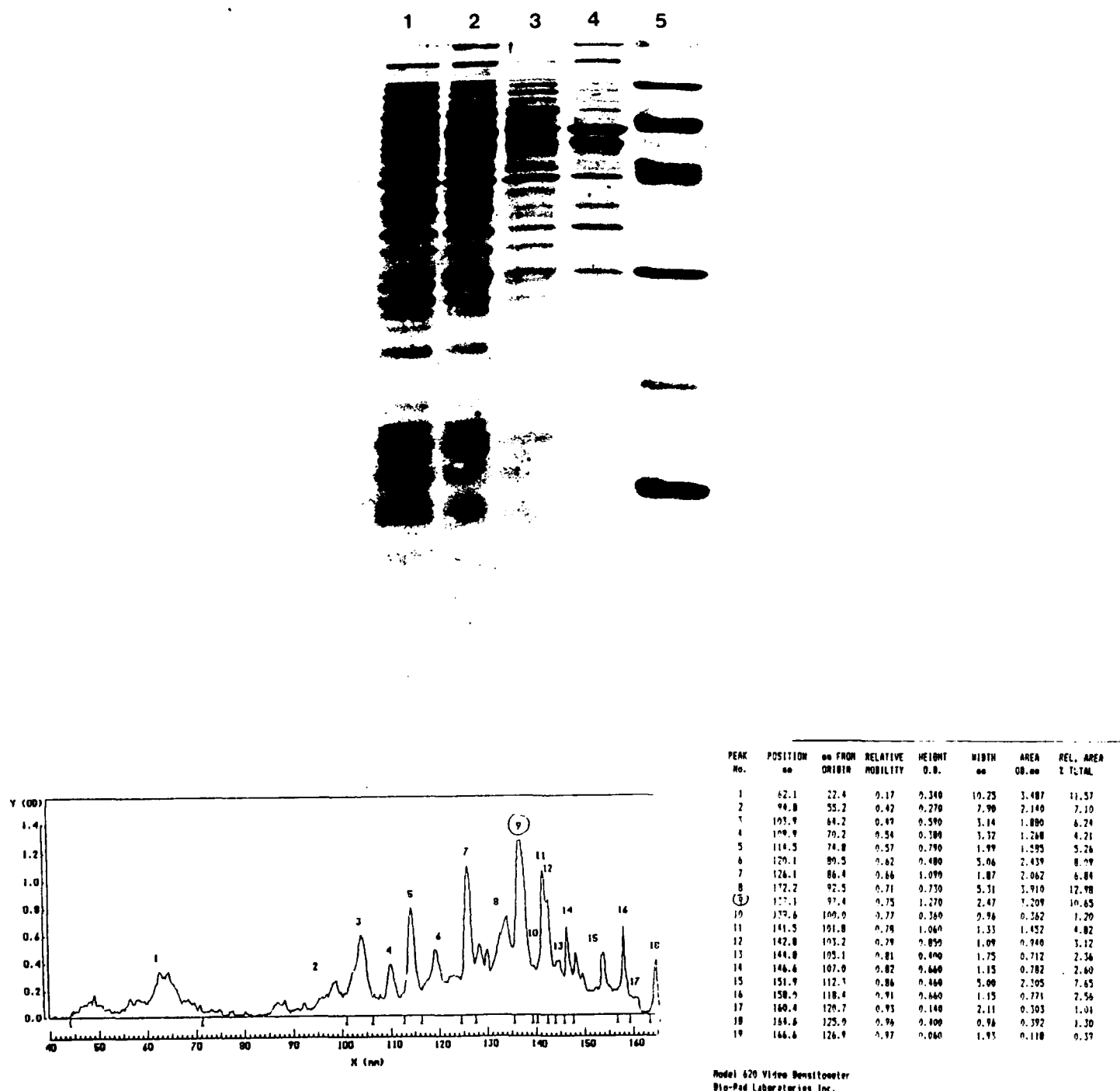


Figure 3:

Top: Expression of rhAChE in the thermoinducible clone A425SpAIF-34. Samples of total lysate and cells disrupted by sonication were analyzed 15% SDS-PAGE. Lane 1: total cell lysate from uninduced culture. 2: total cell lysate from induced culture. 3: soluble fraction of sonicated culture. 4: insoluble fraction of sonicated culture. 5: molecular weight markers; from top to bottom (kD) - 97,66,47,30,20,14.4.

Bottom: Densitometric analysis of lane 2 shown on top. Peak number 9 correspond to the 62kD rhAChE protein band. The numeric display on the right of the scan shows the data and the relative area, as percent, of the entire lane length.

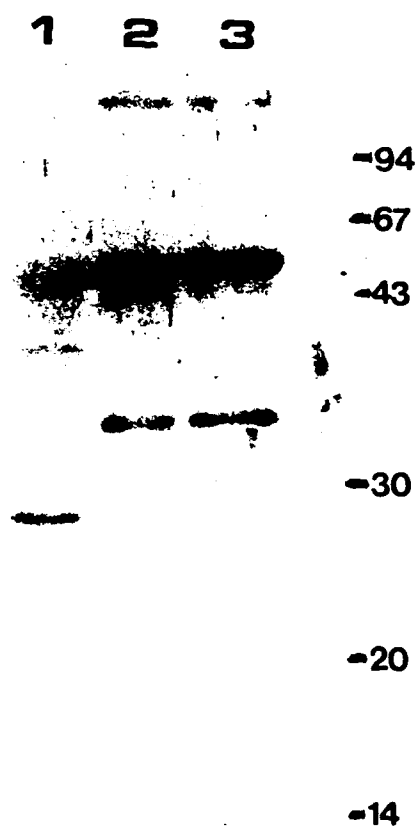


Figure 3A: Western blot of rhAChE. Total cell lysate and inclusion bodies isolated from clone SØ930pMFL-52Ser were subjected to SDS-PAGE on a 15% acrylamide gel, blotted onto nitrocellulose paper and immunoreacted with human erythrocyte anti-AChE. Lane 1: total cell lysate of control (host) SØ930; lane 2: total cell lysate of SØ930pMFL-52Ser; lane 3: inclusion bodies fraction isolate from pMFL-52Ser. The most intense immunoreactive band correspond to the 62-64kD polypeptide of rhAChE. Numbers on right are protein MW markers in kD.



Figure 3B: Expression of rhAChE in clone driven by the constitutive deoP promoter. Lane 1: Total cell lysate of SØ930 host containing no plasmid (control). Lane 2: total cell lysate of SØ930pMFL-52Ser. Lane 3: insoluble fraction of sonicated SØ930pMFL-52Ser. Lane 4: molecular weight markers, from top to bottom (kD) - 97,66,47,30,20 and 14.4.



Figure 3C: Zymogram of rhACh activity on 6% polyacrylamide gel. Lane 1: native rhAChE with Cys⁵⁸⁰ reconstituted from inclusion bodies derived from clone SØ930pAIF-52. Lane 2: mutant rhAChE with Cys⁵⁸⁰-Ser⁵⁸⁰ substitution reconstituted from inclusion bodies derived from clone SØ930pMFL-52Ser.



Figure 4: Affinity purified rhAChE derived from clone SØ930pMFL-52Ser. Lane 1: MW marker proteins 94, 67, 43, 30, 20 and 14kD from top to bottom, respectively; lane 2: pattern of proteins that did not bind to the affinity column MAC-Sepharose 4B; lane 3: the 62-64kD protein of active rhAChE eluted from the affinity column.

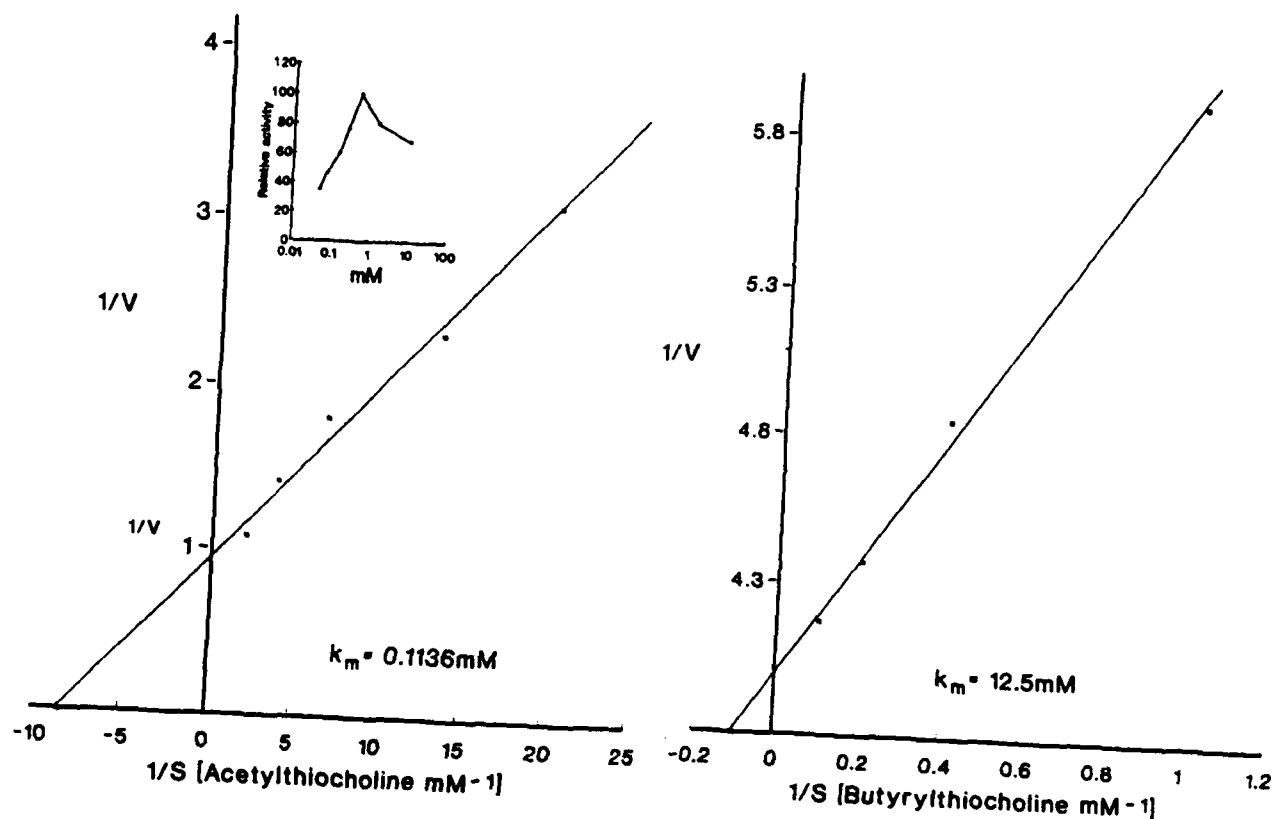


Figure 5: Kinetic parameters of purified rhAChE reconstituted in vitro: double-reciprocal Lineweaver and Burk plot and substrate inhibition (inset). K_m was determined from Lineweaver-Burk plot. Constant volume of highly purified rhAChE derived from clone SØ930pMFL-52Ser was added to the assay mix containing the indicated concentrations of acetylthiocholine or butyrylthiocholine. Activity was monitored in a PU8700 spectrophotometer.

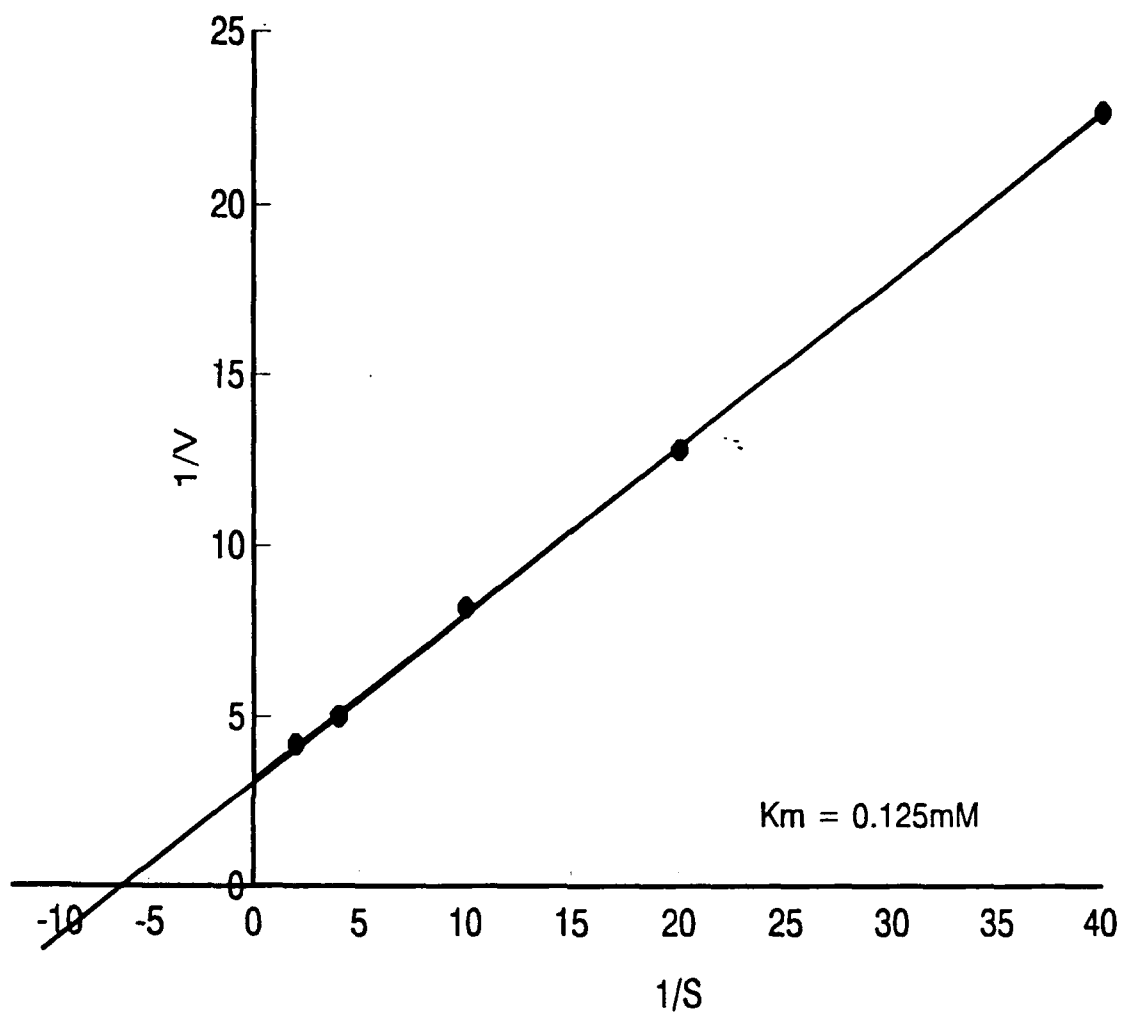


Figure 5A: *K_m determination of rhAChE derived from clone SØ930pAIF-51 which harbors the native amino acid sequence of hAChE catalytic subunit. Crude non-purified active enzyme obtained after in vitro refolding, was used for this determination.*

Inhibition of AChE

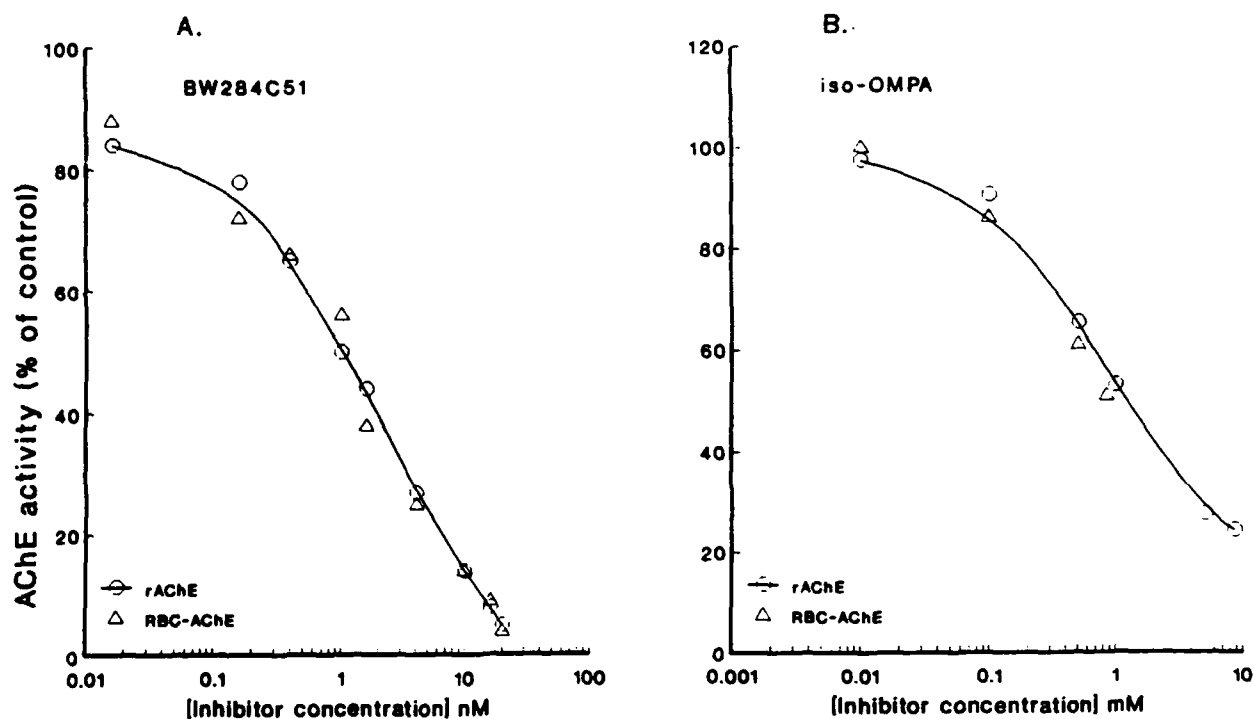


Figure 6: Effect of inhibitors on rhAChE. Highly purified rhAChE (o) derived from clone SØ930pMFL-52Ser and erythrocyte AChE (Δ) obtained from Sigma Co. (St-Louis) were assayed in the presence of BW284C51 (panel A) or iso-OMPA (panel B) at indicated concentrations.

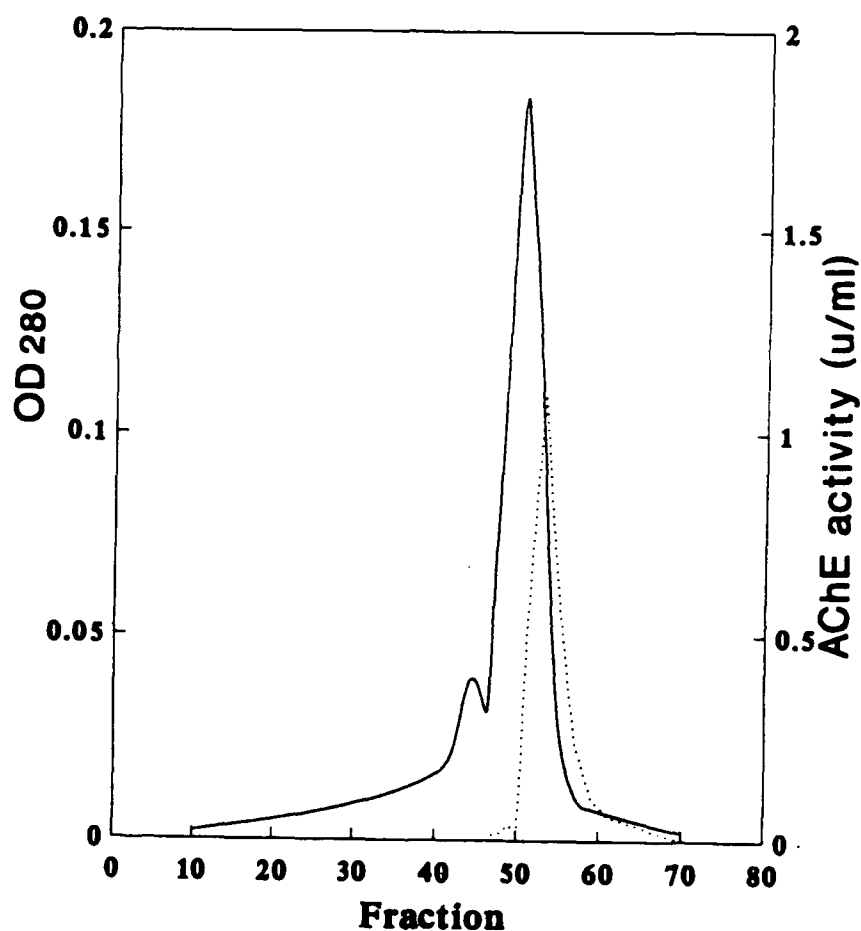


Figure 7: Molecular weight determination of active rhAChE. Purified rhAChE obtained from clone SØ930pMFL-52Ser was mixed with bovine serum albumin (BSA) and subjected to size exclusion chromatography on Sephacryl-300. Absorbance was monitored at 280nm, and AChE activity was determined in each fraction. The solid line shows the elution pattern of BSA containing a minor peak corresponding to a M.W. of 135kD of dimers and the larger peak corresponds to BSA monomer of 67kD. The dotted lines show the elution profile of rhAChE as determined by activity. A total of 8mg of BSA containing 50 units of rhAChE in 18ml was applied to a column of 720mm x 26mm (Pharmacia).

1 CCTCTCTCCCTCATCTTTGCCAACCTGCCCCACCTCTCTGAGCTGAGCGATAACCT
 61 TGGGCGGACAGTGCCTAATCTCTCTCTCTGCTTCTGACCGACCTTCACCTTTT
 121 CTTTCTTTCTCCCGACGACGCGCTGCGCTGACGCAATGAGGCCCCCGCAGTGTCTG
 181 CTGCACACGCTTCCCTGGCTTCCCACTCTCTCTCTCTCTCTGCTCTGCTCTGCTGGA
 241 GGAGTGGGGCTGAGGGCGGGGAGGATGACAGCTGCTGGTACGGTGGCTGGGGCGGG
 301 GTGCGGGGCTTCCCTGAAGACCCCGGGGGCTCTCTCTCTCTCTCTCTGCTGCTGCTG
 361 TTTGCGGAGCCACCTGCGGACCGCTGCTTCTGCGACCGGAGCCCAAGCAGCTTGG
 421 TCAGGGGTGGTAGAGCTACACCTTCCAGAGTGTCTGCTACCAATATGTGGACACCTA
 481 TACCCAGGTTTTGAGGGACCGGAGATGTGGAACCCCAACCGTACGCTGAGCGAGGACTGC
 541 CTGACCTCAACGTGTGGACACCATACCCCGGGCTACATCCCGACCCCTGTCTCTCTC
 601 TGGATCATGGGGGTGGCTTCTACAGTGGGGCTCTCTCTCTGAGCTGTACGATGGCGCG
 661 TTCTGGTACAGCGCGAGAGGAGTGTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 721 GGTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 781 AGGCTGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 841 GTGACGCTGCTTGGGGGAGCGCGGGGAGCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 901 CCGAGCGCGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 961 GCGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1021 GTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1081 CAGCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1141 TTCTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1201 AACCGCGGAGCTTCCACGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1261 TATTTCTGCTTACGGGGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1321 GCGGAGTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1381 GCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1441 GCGCTGAGGATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1501 CGACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1561 CTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1621 ATCCCCCTGGACCCCTCTCGAACTACACGGGACGAGGAGGAGGAGGAGGAGGAGGAGGAGG
 1681 ATGCGATACTGGGCACTTTGCGCGGACAGGGGATCCCAATGAGCCCGGAGACCCCAAG
 1741 GCGCCCAATGGCCCCGTACACGGCGGGGCTCAGCAGTACGTTAGTCTGACCTGCGG
 1801 CCGCTGGAGGTGCGGGCGGGGCTGCGCGCCCGAGGCTGCGGCTTCTGGAACCGCTTCTCTC
 1861 CCCAAATTGCTCAGCGCCACCGACAGCTCGACGAGCGGAGCGGAGCGGAGCGGAGCGGAG
 1921 TTCCACCGCTGAGCTCTCTATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
 1981 CAGGATCGCTGCTCAGACTGTGACCCCGGCGGACCCCGATGCTCTGCTGCTGCTGCTGCTG
 2041 CCCCCAGCTGTATATACTATTTATTTTACGGGCTGGGCTATAACACAGACGAGCCCCAGA
 2101 CTCTGCCATATCCCAACCCACCGGAGCTGCGCGGGGCTGCGGCTCTGCTGCTGCTGCTGCTG
 2161 TTAGGCTGAGCT
 2221 CAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 2253

Figure 8: Nucleotide and amino acid sequence of hAChE as published by Soreq et al (1990). The single letter code was used to denote individual amino acids. See the cited reference for explanation of boxed, underlined sequences and arrows.

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